

⑫

EUROPEAN PATENT APPLICATION

⑰ Application number: **88500077.8**

⑸ Int. Cl.⁴: **A 61 K 39/095**

A 61 K 39/40, C 07 K 15/06,
A 61 K 35/14

⑱ Date of filing: **30.07.88**

③① Priority: **30.07.87 CU 12587**

④③ Date of publication of application:
01.02.89 Bulletin 89/05

⑧④ Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

⑦① Applicant: **CENTRO NACIONAL DE BIOPREPARADOS**
1914, 212 Street Atabey, Playa
La Habana (CU)

⑦② Inventor: **Campa Huergo, Concepcion**
255, N. St. Vedado
La Habana (CU)

Sierra Gonzalez, Victoriano Gustavo
101, San Pedro St. Santa Clara
Villa Clara (CU)

Gutierrez Vazquez, Maria Mercedes
A Street No. 1302 Bejucal
La Habana (CU)

Bisset Jorrin, Gonzalo
563, San Miguel Street Barrio Obrero
La Habana (CU)

Garcia Imla, Luis Guillermo
Mercado Street Barrio Obrero
La Habana (CU)

Puentes Rizo, Gisela de la Caridad
Maria Luisa Street No. 682 Mantilla
La Habana (CU)

Sampedro Herrera, Maria del Carmen
6156, Delicia Street, Lawton
La Habana (CU)

Sotolongo Padron, Franklin
72, Santo Suárez Street
La Habana (CU)

Xochitl le Riverend Morales, Eloisa
31, Loma Avenue Nuevo Vedado
La Habana (CU)

Galguera Dominguez, Manuel Alfredo
9, 9na. Street Santa Clara
Villa Clara (CU)

⑦④ Representative: **Gil Vega, Victor**
Estébanez Calderon, 3 - 5B
E-28020 Madrid (ES)

⑤④ Vaccine against group B Neisseria meningitidis, gammaglobulin and transfer factor.

⑤⑦ A method is provided for obtaining a vaccine against the different pathogenic serotypes of group B Neisseria meningitidis characterized by starting from live microorganisms of any one of the known pathogenic serotypes of the B group without inactivation, from which the extraction of the vesicles of the outer membrane and the protein antigenic complex of 65 - 95 KD molecular weight is carried out using detergent, enzyme and ultrasound combined in the treatment. The resulting product, after treatment to eliminate the nucleic acids, is purified by a dissociating treatment with detergent, ultrasonic bath and column chromatography. The multi-antigenic material so obtained is purified to obtain the protein antigenic complex of 65 - 95 KD molecular weight for HPLC chromatography (TSK 3000 SWG column) or affinity chromatography with monoclonal antibodies, or hydrophobicity chromatography, or ionic exchange chromatography or a combination of any one of them. The protein antigenic complex is then added to the fraction that

contains the vesicles by ultrasound treatment so that it will be anchored on then, in a proportion of 15 per cent \pm 3. The capsular polysaccharide is also added, in a proportion of 1.1 - 1.4 with respect to the protein and the adjuvant in a relation ranging from 2 - 100 mcg/protein mcg. The different components of the mixture may be sterilized by cobalt 60 ionizing radiations with doses from 5 - 25 Kgy and a temperature between 1 - 4 grade C before preparing the mixture or the resulting mixture may be sterilized by this procedure.

EP 0 301 992 A2

Descripti n

"METHOD FOR OBTAINING A VACCINE WITH WIDE PROTECTIVE RANGE AGAINST AGAINST GROUP B NEISSERIA MENINGITIDES, THE RESULTING VACCINE, GAMMAGLOBULIN AND TRANSFER FACTOR"5 Technical field

The present invention is related with the field of medicine and more particularly with the prophylaxis and treatment of diseases caused by pathogenic bacteria.

10 The aim of this invention is to provide a method for obtaining a vaccine against the different serotypes of group B Neisseria meningitidis, and also the resulting vaccine.

Another aim of the present invention is to provide a method for obtaining a preparation of antimeningococcal hyperimmune gammaglobulin and the gammaglobulin so obtained.

15 Finally, an additional aim of the present invention is a method for obtaining a specific transfer factor (dializable factor in leukocyte extracts) which is used for transferring T-cell immunity against Neisseria meningitidis B and the transfer factor.

State of the art

20 Neisseria meningitidis has the typical envelope of Gram-negative germs, consisting of a cytoplasmic membrane, a peptidoglycane layer and an outer membrane with three layers, which, together with the capsular polysaccharide, constitute the bacterial wall.

From an immunologic point of view, those structures which potentially may come into contact with the immune system in any of the stages of the microorganism-host relationship and especially those of the first stages, i.e. the most external components, are of significance. It is not casual that the classification of this microorganism has been made on the basis of groups, types and subtypes, which have been established thanks to reactions with poly clonal (Frasch, C.E. and Chapman, 1973, *J.Infect.Dis.* 127: 149-154) or monoclonal antibodies (Hussein, A., *Monoclonal Antibodies and N. meningitidis*. Proefschrift. Utrecht, 1988).

30 Nowadays 12 serogroups are known: A, B, C, X, Y, Z, 29-E, W-135, H, I, K, and L (Ashton, F.E. et al., 1983, *J.Clin. Microbiol.* 17:722-727; Branham, S.E., 1956, *Can.J.Microbiol.* 2:175-188; Evans, A.C., 1920, *Lab. Bull.* 1245: 43:87; Shao-Qing, et al., 1972, *J.Biol.Stand.* 9: 307-315; Slatore, K.W., 1961, *Anti.v. Leeuwenhoek J Microbiol. Serol.* 29:265-271). Such groups have been differentiated according to the capsular polysaccharide (CPS) which presents differences among each of these group in certain antigenic determinants (Branham, S.E., 1940, *Bacteriol.Rev.* 4:59-96; Frasch, C.E. et al., 1985, *Rev.Infect.Dis.* 7: 504-510).

35 In spite of variations in the epidemiological situation in different regions of the world and the "knowledge gaps" due to a lack of adequate systems of sanitary control in some countries, it has been possible to establish that, among all these groups, at present only groups A, B, C, Y, and W-135 are responsible in the world for more than 90% of the cases (Abdillahi, H., 1988, Proefschrift, pag.13 Utrecht, Nederland), according to other authors they are responsible for more than 95% (Frasch, C.E. *Enr. J. Microbiol.* 4: 533-536).

40 The capsular polysaccharides, purified and characterized, and used so far as vaccines have proved efficient enough to control the outbreaks and epidemics of the groups A, C, Y, and, W-135, as mono-, di-, tri-, or tetravalent vaccines (Gold et al., 1969-1970, *Bull.WHO* 45:272-282; Gotschlich et al., 1969, *J. Exp. Med.* 129:134-136; Hankins, 1982, *Proc. Soc. Biol. Med.* 169: 54-57). In spite of deficiencies still to be solved such as: poor or nonresponse to polysaccharide C in children under 2 years of age and the thermolability of polysaccharide A, besides difficulties regarding the induction of tolerance after re-vaccination with polysaccharides A and C, to the point that in some places increase of cases has been detected (Marzochi, K.B.F. *Meningite Meningococcica*. Tese (doutorado) Universidade Federal do Rio de Janeiro, Brasil (328 pag.), 1985), still although they may not be definitive, now the polysaccharide vaccines are an effective enough weapon in the struggle against disease caused by groups A, C, Y and W-135, but not against group B, the capsular polysaccharide of which is a very poor immunogen (Wyle et al., 1972 *J.Infect. Dis.* 126:514-522; Zollinger, et al., 50 1979, *J. Clin. Invest.* 63: 836-934; Jennings et al., 1981, *J. Immunol.* 127: 104-108). It may be due to different causes such as the cross reactivity with antigens of the host itself (Brain sialoglycopeptides), conformational changes suffered by these polysaccharides when they are purified, and their great sensitivity to enzymatic and other types of degradation occurring in the organism, such as: rapid esterification, etc. (Lifely, M.R. and Moreno, C., *Lancet*, Jan.25, 214-215). The final result is that, despite the intense work of important researchers and companies such as: The Wellcome Fdion., it has been impossible yet to convert the group B polysaccharide into a satisfactory immunogen. Because of the above mentioned reasons, the general solution of the other polysaccharide groups used for the meningococcal disease does not work for group B. This, besides the fact the serogroup B is today the main cause of the meningococcal disease in most temperate countries as well as other regions throughout the world, makes it necessary to obtain alternative vaccines. Every attempt at finding these alternative vaccines should therefore be based upon non-capsular antigens, especially those in the outer membrane, taking into account their significance in the microorganism-host relationship.

60 Among the components of this membrane, almost every work group has focused its attention on the proteins, since the other relevant components would be the Lipopolysaccharides (LPS), but their highly toxic

nature, their high pyrogenic activity and their mitogenic capacity have prevented considering this element as a vaccine option, although it is the most important in the pathogenesis of the meningococcal disease.

The majority proteins in the outer membrane, both from group B and C give rise to subdivisions of these groups in serotypes (from now on we shall only refer to group B). They are based on the immunologic specificity of these majority proteins detected in reactions with the polyclonal and monoclonal antibodies in different techniques (Abdillahi, U Proeschrit, Utrecht, Nederland, 1988). Group B has already been subdivided into more than 18 serotypes.

For this identification use is made of such techniques as double immunodiffusion, counter immunoelectrophoresis, radioimmunoassay, ELISA, SPRIA, coagglutination using protein A coated Staphylococcus aureus to fix monoclonal antibodies or other solid carriers, agglutination by latex, etc. (Gold, R. and Wyle, F.A. 1970, 1:479-484; Jones, O.M. and Tobin, B.M., 1976, J.Clin.Pathol. 29:746-748; Zollinger, W.D. and Mandrell, R.E., 1977, Infect.Immun 5:98-102; Frasc, C.E. and Chapman, S.S., 1973, Infect.Dis. 127: 149-154; Danielsson, D. and Olsen, 1979, J.Clin.Pathol. 32: 136-142).

With the aid of monoclonal antibodies it has even been possible to subdivide further different serotypes correlating them with their degree of "virulence", e.g. serotype 21 was subdivided into 2a, 2b and 2c (Abdillahi, H and Poolman, J.T., 1988, Proefschrift Ch. 6 pag. 69-78, Utrecht, Nederland; Poolman, J.T. et al, 1980, J.Gen.Microbiol 116:465:473).

It is obvious that, by using the majority proteins of the outer membrane as a basis for the immunogen, we are preparing a specific serotype immunogen. In spite of the fact that it has been found that among all serotypes only a few are disease agents (Frasch, C.E., et al., 1972, Seminare in Infections Diseases, Vol.1 S.I.M. Book Corp., N. Y. 304-337) while others seldom are, it would be impossible to prepare an immunogen based on serotype, which would have sufficient antigenic determinants for all the serotypes causing disease. Therefore, serotype vaccines as such have a small range of effectiveness, aiming only at the specific serotype with which the vaccine was elaborated. This has been the history of almost all the protein vaccines prepared in the last few years and that has been among several others, one of their major limitations, vaccines based on serotype 2a proteins have been prepared from vesicles of the outer membrane (Frasch, C.E. et al., 1982, Infect. Immun. 37: 271-280) and certain variants of these vaccines have been studied regarding their immunogenicity and toxicity in animals (Pepler et al., 1982, Infect. Immun. 37: 264-270) and assessed for their safety and immunogenicity in voluntary adults (Frasch, C.E. et al, 1982, Sem.Infect Dis., 4 Zollinger et al, 1979, J. Clin Invert 63: 836-884).

Other research work also reveals the importance of the solubility of these vaccines (Frasch, C.E. et al., 1982) Sem. Infect Dis, 4; Frasc, C.E., 1978, J. Exp. Med. 147:629-644).

The benefits of the use of capsular polysaccharides and different adjuvants of aluminum hydroxide or phosphate have also been reported by numerous authors (Frasch, C.E., 1983, Med. Microbiol. Vol. 2 Acad. Press, N.Y.) In some cases a careful study has been carried out on the correlations among the proportions of those components, their electron microscopy picture and their response in different animals, including primates and humans of different age groups (Campa, C. et al., 1986).

In our research work (Campa, C. et al., prepared for publications) we even found the solution for the lack of immunogenicity in children under 2 years of age.

Summary of the invention.

The present invention provides a method for the obtention of a vaccine against different pathogenic serotypes of group B *Neisseria meningitidis*, whose main novelty is based on the use of a protein antigenic complex of high molecular weight common to all pathogenic serotypes, which anchored in the vesicles of the outer membrane of the microorganism, constituted, among other components, by serotype majority proteins of the strain used in the production of the whole adjuvanted, permits the obtainment of a protective preparation never before attained.

The vesicles constitute one of the elements of the vaccine that contribute antigenic determinants of the antigenic majority proteins, they regulate immunologically LPS and constitute the ideal structure for anchorage of other proteins, which are in minority in the B meningococcus, but that are common to all the pathogenic types tested and very good immunogens, that induce long-lasting bactericidal response.

The wide range common protective character is conferred by the antigenic community in group B in the zone of the high molecular weight protein antigenic complex. The high molecular weight protein antigenic complex also "adjuvates" and increases the response against other antigens, besides responsible for the prolonged immunologic memory.

The serotype antigens are important in the first stage of the protective response (4-6 months), but afterwards the protective titer is mainly detected against the high molecular weight protein antigenic complex.

For this reason the second component of our vaccine is the protein antigenic complex of 65-95 KD molecular weight, which is added to the vesicles in a proportion of 15% \pm 3.

The vesicles by themselves would probably serve as a serotype vaccine of moderate and transitory effectiveness.

Another novelty of the method is the extraction of the proteins of the outer membrane by combined treatment of live microorganisms with detergent, enzyme and ultrasonic bath, and more specifically using sodium doxylcholate, sodium dodecyl sulphate and Brij-96 as detergents of the following respective

concentrations: lower than 1%, from 0.1-5%, and from 0.01-0.05%, all are independently combined with lysozyme at a concentration of 0.03-1% and ultrasonic bath. A variant of this process of extraction consists in using Tween 80 combined with ultrasonic bath.

5 Examples of membrane protein extractive media.

10	EXTRACTIVE MEDIA	YIELD	Contam.DNA
	DOC 0.1-2%	2-3	20%
	DOC less than 0.1% + lysozyme 0.03% + US	2-3 mg/g	20%
15	Tween 80 0.1%	1,2-2 mg/g	15%
	SDS 0.1-0.5% + lysozyme 0.03-1% + US	1,8-3,2 mg/g	22%
20	Brij-96 0.1-0.5% + lysozyme 0.03-1% + US	1.5-2.1 mg/g	12%

US: ultrasonic bath. Contaminant DNA refers to the % of the total of DNA/
Total protein which must be eliminated in the subsequent steps.

The extraction process of membrane proteins is carried out at a temperature between 2-10°C, with an extraction time between 5 minutes and 5 hours with magnetic or mechanical (250-950 rpm) stirring; the medium in which extraction takes place may be constituted by different aqueous buffers and pH is maintained between 6.5-9.0 depending on the extractive combination used; magnetic or mechanical stirring can be alternated with ultrasonic treatment.

The method object of the present invention also possesses as a novel characteristic the procedure for purifying the protein extract by a dissociative treatment with detergent, ultrasonic treatment and chromatography, after the elimination of nucleic acids. With this combination the modulation of the content of LPS, phospholipids, and other lipids of the vesicles, which provides the vaccine with the capacity to induce an effective antibody response against endotoxin. As detergent sodium deoxycholate, Brij-96, Tween 20, Tween 80 in a concentration of 0.1-6%, the molecular sieving is carried out in a Sephacryl S-300 and S-400 or Sepharose C1-4 column.

It is still another novel characteristic of the method of the present invention, that the purification of the antigenic material resulting from the modulation to obtain the high molecular weight protein antigenic complex of 65-95 KD molecular weight, using HPLC chromatography (TSK3000 SW6 column), or affinity chromatography with monoclonal antibodies or hydrophobic chromatography or ionic exchange chromatography or a combination of any of these.

It is an essential feature of this method that it adds to the fraction containing the vesicles, of the protein antigenic complex in a proportion of 15% \pm 3 using ultrasonic treatment so that the complex is anchored on them.

With ultrasonic treatment integration of the antigenic complex is facilitated, onto the vesicles naturally formed and that already possess traces of those proteins as leftover from purification; in this way the vesicle works as a carrier or adjuvant with respect to that antigen of high molecular weight.

To the fraction containing the vesicles capsular polysaccharide is added in a proportion of 1:1-1;4 with respect to protein and adjuvant in a proportion of 20-100 mcg/mcg of protein. As adjuvant aluminum hydroxide, aluminum phosphate or calcium phosphate.

By adding the aforementioned proportions in which capsular polysaccharide and adjuvant an increase in the immunogenicity of the vaccine preparation is obtained, and also an adequate presentation of the lipopolysaccharide component, which in this way has reduced capacity to induce undesirable reactions to a minimal level.

The different components of the vaccine mixture are radiation sterilized by ionizing cobalt 60 radiations, with 5-25 Kgy doses, at a temperature from 1-4°C before preparing the final mixture or sterilization may be carried out by this procedure once the resulting mixture is obtained. the use of ionizing radiation for sterilization of the individual components of the vaccine mixture or jointly, in liquid or lyophilized form for this type of vaccines.

It is a variant of this stage of the method, according to the present invention, the sterilization of the components is effected by membrane filtration.

It is a variant of the sterilization stage the use of both procedures in combination: cobalt 60 ionizing radiation at mentioned doses and temperatures and membrane filtration, in such a way that some of the components

are sterilized by the first procedure independently and the rest by the second described procedure.

The vaccine so obtained by the described method possesses a wide longlasting protective range against the various pathogenic serotypes of group B *Neisseria meningitidis*. It is the first vaccine against B meningococci to reach the level of mass efficacy field trial, having already reached a total of 3000 000 voluntary vaccinees.

This vaccine contains an immunologically effective quantity of the protein antigenic complex or molecular weight 65-95 KD, which confers antigenic community for the different pathogenic serotypes and induces the production of bactericidal antibodies; it also contains a quantity of vesicles in the necessary proportion to induce a strong antibody response against the serotype antigenic determinants and the endotoxin; a proportion of capsular polysaccharide, which encreases the solubility and immunogenicity of the whole, which at the same time, increases the response of the organism to the polysaccharide component, even for children under 2 years of age and defines its polyvalent property. The vaccine is optimized by the necessary quantity of adjuvant.

The invention also permits to obtain, from blood donations of the vaccinees, of a hyperimmune antimeningococcic gammaglobulin preparation used successfully for the first time in the prophylaxis and treatment of meningitis caused by any or the various pathogenic serotypes of group B *Neisseria meningitidis* against which it possesses bactericidal and neutralizing activity.

The invention also provides the obtainment, for the first time, of a specific transfer factor (dializable factor from donor's white blood cells), which is capable of transferring T-cell immunity against this microorganism.

Controls.

The controls of polysaccharide C are established by the standards of the WHO and they are strictly met. The controls for the protein preparations are:

Electrophoresis in polyacrilamide gel (Tsai and Frasch, 1980, *J.Bacteriol.* 141: 169176). In the electrophoretic patterns obtained, besides the serotype majority proteins, 12-15% proteins of the antigenic complex with high molecular weight 65-95 KD are found.

KDO. (Osborn, et al., 1963, *Proc. Natl. Acad. Sci. USA* 50: 499-506). In the variant studied up to field level the permissible limit established was 10%.

Other variants with KDO over 10% are now being studied in human volunteers.

Nucleic acids. After eliminating protein with extensive phenol and dialysis, differential spectrophotometric reading (260-280 nm) is carried out so as to verify detectable absence of proteins and then the nucleic acid concentration is calculated by light adsorption at 260 nm, using the following extinction coefficient:

$$\begin{array}{l} 0.1\% \\ E \qquad \qquad = 20.0 \\ 1 \text{ cm} \end{array}$$

Sialic acid: the residual polysaccharide B content was measured according to the method of L. Svennerholm (*Biochem. Biophys. Proc.* 24: 604, 1957). In our variant, conducted up to the field trial level, a 1-10% limit was established.

Electron microscopy. It is carried out as a routine procedure as described by Frasch and Peppler (*Infect. Immun.* 37: 271-280, 1982). The ultramicroscopic image is correlated with solubility and immunogenicity. Other structural studies are under way with the variants under study.

The protein concentration was determined by the Lowry method (1951, *J. Biol. Chem.* 192: 265-275).

Final vaccine controls.

Figures stated below refer to the variant of our vaccine which is undergoing field trial in human subjects.

Composition.

Protein antigens 100 ± 20 mcg per ml. Polysaccharide: 100 ± 20 mcg per ml. Aluminum hydroxide (gel) 4 mg per ml. Thimerosal: 1 g in 10 000 ml. The dose is 0.5 ml.

Thimerosal.

(merthiolate, sodium -ethyl-mercuri-thiosalicylate). Is determined by spectrophotometry using diphenyl-thiocarbzone, according to WHO Manual BLC/UNDP/77.1 Rev. 1 pp 84-85. Permissible limits range from 0.005 to 0.02%.

pH Measurement.

Using all pH meter with glass and calomel electrode and standard buffer solutions (BDH) at pH values 5.0, 7.0, 9.0. Allowed pH values must be in the range of 7.0 ± 0.4 .

Determination of aluminum content:

It is performed according to Appendix D.18 of booklet WHO/UNDP/77/1 Rev. 1 pp 87-88.
The criterion of acceptability is no to exceed 1.25 mg Al (aluminum) per dose.

Determination of antigen concentrations and percent of absorption to adjuvant gel.

The absorbed vaccine centrifuged and concentrations of protein and polysaccharides in the supernatant and sediment are determined using the above mentioned methods.

The preparation is accepted if not over 20% of total antigens originally added are free in the supernatant.

Sterility control.

It is performed according to specifications in Appendix D.27 of BLG/UNDP/77.1 Rev. 1, General Requirements for the Sterility of Biological Substances and the corresponding Cuban standards.

Innocuousness control.

Every batch is tested for innocuousness by giving a human dose (max. 1 ml) to 5 adult mice weighing 17-22 g and not more than 15 ml to 2 guinea pigs weighing 250-350 g.

The preparation is accepted if no animal shows ill health during 7 days following inoculation. If anyone of the animals dies or shows ill health and the others are not affected, the test is repeated. The batch is approved if none of the animals in the second group dies or shows ill health after 7 day

Electron microscopy.

Samples of final vaccine batches are subjected to electron microscop using the method already mentioned.

ELISA:

The solid phase immunoenzimatic assay was performed as described by Peppler and Frasch (Infect. Immun. 37: 264 = 270, 1982), and adapted to our antigen's coupling characteristics, mostly in order to have a reference system for our method (Sierra, et al., 1988. Rev. Cub. Inf. Biot., in press).

This system is based on the use of PVC-microplates with wells covered by polystyrene, and 10 mcl effective volume capacity, which are read in the equipment (SUMA) developed at the Centro de inmunoensay (Habana, Cuba by FernándezYero and colleagues, consisting of a fast reading vertical spectrophotometer-fluorimeter with automated operation and interpretation of results and the 96-position multipipettes, which enable evaluation of thousands of samples in a very short time and facilitates mathematical processing.

This system was calibrated in order to evaluate antibody response in the various vaccine experiments.

Bactericidal microassay.

Slightly modified bactericidal microassay of Frasch and Robbins (J. Ecp. Med. 147: 229-244; 1978) with improvement of the source of complement by obtaining it from SPF rabbit sera (produced at the Centro de Producción de Animales de Laboratorio, Habana, Cuba) for assaying sera from immunized mice; and from fresh blood bank donations, when assaying human or monkey sera.

Statistical analysis.

Non-parametric methods were used in the statistical analysis as distributions of ELISA and bactericidal values did not have a normal pattern, but had some asymmetry.

The Wilcoxon test for independent sample was mainly used. To obtain a better analysis a wide series of percentiles was calculated thus using another approach to the median of each studied group.

Animal tests.

Tests in mice: each variant of the vaccine preparation was submitted to immunogenicity and bactericidal tests in mice (besides the above mentioned safety tests in guinea pigs and mice). The results showed that the preparations are immunogenic and can evoke significant levels of bactericidal antibodies.

Tests in monkeys:

Vaccine variants that were to be used in human volunteers were also tried in monkeys (*Macacuss aethiopiens*) in various doses and schedules. The results showed that they were innocuous, immunogenic and highly capable of eliciting bactericidal antibodies when compared with the placebo.

5

Test in humans:

Reactogenicity tests were carried out in groups of 3, 10 and 30 persons to cover the various doses that were to be assayed; then larger groups were used for immunogenicity tests.

10

The first groups were closely observed by specialised medical personnel during the initial days, studies included: a) vital signs (pulse, temperature, blood pressure); b) local reactions; c) systemic reactions; d) renal function; e) liver function; f) hematologic study (differential count, platelets, coagulograms); g) higher nervous function (EEG); h) cardiac function (ECG).

None of the studies revealed any pathologic changes in any organ or system with any of the doses studied: ranging from 20 to 100 mcg of protein antigens and the other corresponding components.

15

The only reactions observed in most of the volunteers were:

1.- Slight pain at the infection site, especially during the first 48 hours.

2.- Reddening of the infection site and surrounding area.

3.- Low fever, with a mean of 37.5° C.

20

4.- Malaise and headache only in a few subjects, very transitory, within the first 10-12 hours.

No serious or remarkable reactions occurred, no immediate or delayed hypersensitivity was observed.

The blood count was slightly changed for neutrophils and significantly for lymphocytes.

Each volunteer was given a printed form to be filled with their observations. A part of the printed form was to be filled by the medical personnel. This practice has been maintained for the reactogenicity test of each batch produced and released for use.

25

Immunogenicity tests and induction of bactericidal antibodies in human subjects.

Dose response studies were carried out in human groups of 100, 200, 300, 500 and 1000 voluntary adults (20 - 100 mcg) and for determination of vaccination schedules, dose intervals and number of doses. Tests were conducted first in young adults and then with controlled groups of different ages and social background in numbers that gradually increased (3, 10, 30 and 50 subjects).

30

A. Adults aged 18 to 45 years.

B. Students of both sexes and different educational systems (boarding and non-boarding students) aged 13 to 18.

35

C. Primary school children aged 6 to 12.

D. Children aged 2 to 6 years.

E. Infants aged 6 months to 2 years.

F. Infants aged 3 to 6 months.

40

G. Adults aged over 45 years.

Subjects under 12 years of age did not receive doses over 50 mcg.

The results were highly satisfactory. No undesirable reactions were observed except for those mentioned. In children those reactions were less intense as age decreased.

Doses of 20 to 100 mcg proved significantly immunogenic, but the best results were obtained with 50 mcg. This dose was selected from all ages, with the already described variant, which reached field evaluation. All these studies, including those with animals, reactogenicity, etc. were compared with control groups which were given placebo, having the same appearance, but lacking the active components, that is, aluminum hydroxide only.

45

Antibody measurements by ELISA and bactericidal tests were made as already mentioned (*vide supra*).

50

The responses of various antibody types and subtypes were studied, the most consistently important response being that of Ig G (immunoglobulin G).

For the protection field trial use was made of the variants with the already stated characteristics (less than 10% LPS, 50 mcg protein, 50 mcg polysaccharide C adjuvated with 2 mg aluminum hydroxide). The other variants are being studied in even smaller groups.

55

Schedules of vaccination selected for the field trial.

Although other schedules using 2 and 5 doses and different intervals are being studied, the one selected was two separate doses with an interval of 6-8 weeks.

60

Study of hyperimmune gammaglobulin obtained from vaccinated volunteers.

Purification of the plasma Ig G fraction was undertaken using the modified method of E.J. Cohn et al. (*J. Am. Chem. Soc.* 68: 459-476, 1946) from blood donations of 250-350 ml, the extractions having been made more

65

than 4 weeks after vaccination, checking the quality of individual responses (ELISA and bactericidal microassay). The Ig G fraction thus obtained with over 90% purity was submitted to all controls oriented by WHO concerning the use of endovenous preparations (W.H.O. Tech. Rep. Ser. 567, 1975, idem. 610, 1977), including tests for AIDS and hepatitis viruses and the confirmation of its content of specific and bactericidal antibodies against the circulating strains. The process considerably enhanced the specific antimeningococcal activity of the preparation as compared with the initial sera. It showed high concentration of specific antibodies against the various antigens of the vaccine preparation and of the native strains and high bactericidal titers against these. Following the scientific and legal procedures its use was sanctioned for children with meningitis and/or meningococcaemia, its use having proved highly effective; its antipyretic effect and its influence in the general state and evolution of patients are specially remarkable. This hyperimmune gammaglobulin is now being produced on a higher scale for distribution to intensive care units throughout the country.

The action of this preparation is due not only to its bactericidal characteristics but also to the possibility of removing various antigens released by lysis from meningococci and whole meningococci (Campa, C. and Sierra, G., *Rev. Ciencia* 2, Julio, 1988; Galguera, M. et al., *Rev. Cub. Hemat. Immun. Hemot.* 2, 1988).

The effect of this hyperimmune antimeningococcal gammaglobulin obtained from vaccinees in the treatment of patients with meningitis or meningococcaemia caused by B meningococci is another indirect proof of the vaccine effectivity.

Other results with the vaccine variant sterilized by cobalt 60 ionizing radiations.

An extense and intense physico-chemical and immunologic characterization of the irradiated preparation was carried out, including studies of teratogenicity, carcinogenicity, mutagenicity, determination of new compounds, and repetition of all the obligatory tests in animals and human volunteers for reactogenicity and immunogenicity.

Finally, the batches of the selected variant with all the control passed satisfactorily were used in a group of 50 young adult volunteers to obtain and evaluate hyperimmune gammaglobulin, which was produced, resulting of the same quality in all its parameters and clinical use to the one obtained from the variant vaccines used in the effectiveness field trial.

Effectiveness field test.

A number of variants of our wide range vaccine are being tested in variants stages in human test. The most advanced variant among these is the one selected for the field trial.

Main characteristics of the vaccine used in the field trial.

1. Protein antigen: 60-75%; majority serotype proteins 12-18%; high molecular weight antigenic complex, 65-95 KD.
- Rest; low and high molecular weight contaminants.
2. Polysaccharide C: 50 mcg per dose.
3. A1(OH)3 gel: 2 mg A1(OH)3 per dose; lower than 1.25 mg A1 per dose.
4. pH: 7.0 \pm 0.4
5. Gel adsorption: Higher than 80% for the vaccine antigens.,
6. LPS: Lower than 10%.
7. Polysaccharide B: Lower than 5%.
8. Nucleic acid: Lower than 10%.
9. Thiomersal: 0.005 to 0.02%.
10. Stability: Over 2 years at 2 - 8°C.

Immunobiologic characteristics.

1. The presence of the high molecular weight antigenic complex, with bands common to all B serotypes tested, and the final arrangement of all these antigens on the vesicle of majority proteins and LPS confer the property of eliciting antibodies not only against the serotype from which the majority proteins were extracted, but also against quite different strains which have in common only some antigen of the high molecular weight complex.

Examples:

Strain / Type		Bactericidal titer
Cuba 385	B4 P1:15	1/64 - 1/256
Norway 44/76	815 P1:16	1/32 - 1/256
USA B2		1/32 - 1/128

2. Four to six months after the second dose there is a sensitive drop of the antibodies to the majority proteins by ELISA and Bactericidal Assay; the antibodies to the high molecular weight antigen complex remain. This was verified by using vaccine variants with different compositions and Western-Blotting studies.

3. Stability of the vesicular complex and its immunogenicity as well as its immunologic memory are increased as high molecular weight component is increased.

Two effectiveness trials are being carried out in the field:

A. Mass vaccination of the highest risk population aged 6 months to 24 years in a whole province. The province with the highest historical incidence, a 30 per 100 000 rate, was selected, and 145 000 persons were vaccinated. No other type of vaccine or placebo was used in this study; several studies are being made on different social and age groups, the results of which will be compared with the historical incidence and the non-vaccinated population. The graph shows the initial months of the campaign and the behavior of the disease.

As can be seen, the epidemic has been cut down in that province and now comparing the total number of cases in each province, where one can observe that the one with the highest rate in the past is not so any more.

B. The principal trial, according to the requirements for the approval of new vaccines, is a placebo-vaccine double blind trial including a total 106 000 boarding students in 7 high incidence provinces. The populations receiving either vaccine or placebo were larger than necessary so that after more than one year - which is the time since the beginning of the experience-the normal losses in such large and mobile populations as boarding students the required figures should be available.

For further details on the planning of this study of effectiveness in the field, reference is made to an official document issued by Bureau of Epidemiology of the Cuban Ministry of Public Health, "Estudio de campo de la vacuna antimeningocócica de proteína de membrana externa y polisacárido C", 14 November 1986, Havana.

Results of the first evaluation of the double-blind PLACEBO - VACCINE experiment

Several months are still necessary before the final evaluation of the effectiveness, but now it can be predicted from the seroconversion levels by ELISA and Bactericidal Test and the results of the therapeutic use of the hyperimmune Gamma Globulin obtained from the vaccinees.

PARTIAL EVALUATION

		Seroconversion (%)	
Group	Total to date	ELISA	BACTERICIDAL TEST
Vaccinees	52 966	90	70
Placebo	53 285	14	12
Total	106 251		

For these studies a representative sample of 2100 boarding students representing the universe was used.

For these studies a representative sample of 2100 boarding students representing the universe was used. The correlation of the carrier state before vaccination was analysed as a possible factor coincident with null or atypical responses to the vaccine. Both the above results and those of the application of the antimeningococcal hyperimmune gamma globulin allow to predict that we have a generation of group B antimeningococcal vaccine, which is superior to previous ones and for the first time effective and susceptible

of being scaled-up.

Detailed description of the invention.

The initial strain in the present invention may in principle be any pathogenic strain of B meningococci circulating or responsible for the majority of the disease in the region where vaccine protection is sought.

This strain is adequately characterized, specially as refers to stability and growth requirements in selected culture media.

The microorganism is adequately cultivated and centrifuged to obtain 500 g (wet weight) of biomass of group B *Neisseria meningitidis*, which is resuspended in 2500 ml of a sodium deoxycholate (0,09% sodium deoxycholate, 50 mM Tris-HCl pH = 8,5, 2 mM EDTA) to which 0,05% lysozyme is added. The extraction process is carried out during 2 1/2 hours at 4°C, during which period of time 10 treatment courses of 30 seconds ultrasonic bath are given, alternating with magnetic stirring at 250 rpm.

The cell debris is separated from the extract by centrifugation at 10 000 g. The supernatant is treated with DNase and RNase at a concentration of 5 mg per extract liter each at 37°C.

The extract is centrifuged at 100 000 g during 2 hours, the sediment is suspended in 200 ml 5% deoxycholate buffer in Tris-EDTA, PH 8-9. Chromatography is performed by molecular sieving in a matrix of Sephacryl S-300 with 1% deoxycholate buffer for elution purposes. The first peak is collected and monitored on the Uvicord at 280 nm.

The first peak eluted from this fractioning is the basic material containing the vesicles, to which the high molecular weight antigenic complex is added in a proportion of 12-18%. This complex is extracted by enrichment of the fraction of 65-95 KD from the first peak by repeating the ultrasonic treatment in presence of 5% Tris-EDTA deoxycholate buffer, followed by an affinity chromatography in a column with anti- P1 and anti-P3 monoclonal antibodies, concentration by ultrafiltration and final chromatography on a preparative column (TSK 300-SWG) with Tri-EDTA buffer.

The protein preparation which contains 60-75% of serotype proteins and 12-1% of high molecular weight antigenic complex, is precipitated and washed in ethanol, after previous sterile filtration on Sartorius type membrane. Maintaining the sterility conditions capsular polysaccharide is added in a 1:1 polysaccharide-protein proportion. This group C polysaccharide was produced according to Gold et al., 1969-1970, WHO Bull. 45: 279-282 and Gotschlich et al., 1969, *J.Exp. Med.* 129: 1349-1365) and sterilized by membrane filtration (*vide supra*).

Finally, to the protein-polysaccharide mixture aluminum hydroxide is added as adjuvant in a proportion of 2 mg of Al(OH)₃ for each 50 mcg of protein and polysaccharide, amounts representing the contents of one dose. The adjuvant gel is previously radiosterilized using cobalt 60 irradiation with a dose of 15 Kgy at a temperature of 4°C.

A final pH of the preparation of 7.0 ± 0.4, an adsorption to the gel of more than 80% of the total protein and polysaccharide, and a content of 10% LPS are guaranteed.

As preservative thimerosal is used at a concentration of 0.005-0.02%.

The whole product is subjected to a battery of physico-chemical and biological controls described in the patent.

It is understood that the description and examples as well as embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

1.-Method for obtaining a vaccine against the different pathogenic serotypes of group B *Neisseria meningitidis* characterized by starting from live microorganisms of any one of the known pathogenic serotypes of the B group without inactivation, from which the extraction of the vesicles of the outer membrane and the protein antigenic complex of 65 - 95 KD molecular weight is carried out using detergent, enzyme and ultrasound combined in the treatment, the resulting product, after treatment to eliminate the nucleic acids, is purified by a dissociating treatment with detergent, ultrasonic bath and column chromatography, the multi-antigenic material so obtained is purified to obtain the protein antigenic complex of 65 - 95 KD molecular weight for HPLC chromatography (TSK 3000 SWG column) or affinity chromatography with monoclonal antibodies, or hydrophobicity chromatography, or ionic exchange chromatography or a combination of any one of them, the protein antigenic complex is added to the fraction that contains the vesicles by ultrasound treatment so that it will be anchored on them, in a proportion of 15 per cent ± 3, the capsular polysaccharide is also added, in a portion of 1.1 - 1.4 with respect to the protein and the adjuvant in a relation ranging from 20 - 100 mcg/protein mcg, the different components of the mixture may be sterilized by cobalt 60 ionizing radiations with doses from 5 - 25 Kgy and a temperature between 1 - 4 grade C before preparing the mixture or the resulting mixture may be sterilized by this procedure.

2.- Method for obtaining a vaccine against the different pathogenic serotypes of group B *Neisseria meningitidis* as in claim No. 1, characterized in that the extraction of protein from the outer membrane of any one of the known pathogenic serotypes of the group B is carried out by a treatment with deoxycholate as detergent in a concentration less than 0.1 per cent W/V, lysozyme at 0.03 - 1 per cent and ultrasonic bath.

3.- Method for obtaining a vaccine against the different pathogenic serotypes of group B *Neisseria meningitidis* as in claim No. 1, characterized in that the extraction of proteins of the outer membrane of any one of the known pathogenic serotypes of group B is carried out by means of a treatment with a sodium dodecyl sulphate (SDS) in a concentration between 0.1 - 5 per cent, lysozyme between 0.03 - 1 per cent and ultrasonic bath.

4.- Method for obtaining a vaccine against the different pathogenic serotypes of group B *Neisseria meningitidis* as in claim No. 1, characterized in that the extraction of proteins of the outer membrane of any one of the known pathogenic serotypes of group B is carried out by means of a treatment with Brij 96 as detergent in a concentration between 0.01 - 0.5 per cent, lysozyme from 0.03 - 1 per cent and with ultrasonic bath.

5.- Method for obtaining a vaccine as in claim No. 1, characterized in that the extraction of protein from the outer membrane of any one of the known pathogenic serotypes of group B, according to a variant of it, using Tween 80 as detergent in a concentration ranging from 0.1 - 2 per cent.

6.- Method for obtaining a vaccine as in claims Nos. 1 to 5 characterized in that the extraction procedure of protein from the membrane is carried out at a temperature between 2 - 10 °C with an extraction time ranging from 15 minutes to 5 hours in a buffered aqueous medium, according to the extraction procedure used at a pH between 6.5 - 9.0 and with magnetic or mechanical stirring at 250 - 950 r.p.m.

7.- Method for obtaining a vaccine as in claims Nos 1 to 6, characterized in that the purification of the resulting products free of nucleic acids, is carried out modulating its content in lipopolysaccharides and phospholipids by means of a dissociating treatment with sodium deoxycholate, or Brij 96, or Tween 20 or Tween 80 as detergents in concentration between 0.6 to 6 per cent and an ultrasound treatment followed by molecular sieving in a Sephacryl S-300 or S-400 or Sepharose C1 - 4B column.

8.- Method for obtaining a vaccine according to precedent characterized in that the different components of the resulting vaccine mixture are sterilized by membrane filtration.

9.- Method for obtaining a vaccine as in claims Nos 1 to 8, characterized in that some of the components of the vaccine mixture are radiosterilized and the rest are sterilized by membrane filtration.

10.- Method for obtaining a vaccine against the different pathogenic serotypes of group B of the *Neisseria meningitidis* as in claims Nos 1 to 9, characterized in that the starting material is a concentrate of the culture supernatant.

11.- Vaccine with wide long-lasting protective range against different pathogenic serotypes of group B *Neisseria meningitidis*, which contains an immunologically effective quantity of the protein antigenic complex of 65 - 95 KD molecular weight, that confers antigenic community in presence of different known pathogenic serotypes and induce the formation of bactericidal antibodies a quantity of vesicles in the necessary proportions that induce a strong response of specific antibodies to the antigenic serotype determinants and to the endotoxin, a capsular polysaccharide proportion that increases solubility and immunogenicity of the whole, which also increases the response to the polysaccharide component, even in children under 2 years of age and defines its polyvalent property and whose presentation is optimized by the necessary quantity of adjuvant.

12.- Vaccine with wide protective range against the different pathogenic serotypes of group B *Neisseria meningitidis* as in claim No. 1, characterized in that it is obtained by method as herein described in claims Nos. 1 to 10.

13.- Antimeningococcal hyperimmune gammaglobulin with wide and effective range for the prophylaxis and treatment of meningitis and meningococemia caused by any of the various pathogenic serotypes of group B *Neisseria meningitidis* against which it possesses bactericidal and neutralizing activity.

14.- Method for obtaining antimeningococcal hyperimmune gammaglobulin characterized in that plasma from adults vaccinated with the vaccine described in claims Nos. 11 and 12 is used as starting material from which their G fraction is obtained.

15.- Specific transfer factor (dialyzable factor from leukocyte extract) which is used to transfer T-cell immunity against group B *Neisseria meningitidis*.

16.- Method for obtaining the B antimeningococcal specific transfer factor characterized in that the starting material used consists of leukocytes purified from blood of adults vaccinated with the vaccine described in claims Nos. 11 and 12.

HISTORICAL AND PRESENT BEHAVIOUR OF THE MENINGODISEASE
AT THE SITE OF THE FIELD TRIAL
(142 000 VACCINEES FROM 6 MONTHS TO 24 YEARS OF AGE

